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Sex-Specific Control and Tuning of the Pattern Generator for Courtship Song in *Drosophila*

J. Dylan Clyne[1] and Gero Miesenböck[1,2,*]
[1]Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA
[2]Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford, OX1 3PT, United Kingdom
*Correspondence: gero.miesenboeck@dpag.ox.ac.uk
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SUMMARY

The differentially spliced transcription factors encoded by the *fruitless* (*fru*) gene are key determinants of sexual behavior in *Drosophila*. They are expressed in a minority of neurons with limited dimorphisms and regulate neural processes that remain largely unknown. Here, we use light-activated ion channels to stimulate *fru*-expressing neurons in the thoracic-abdominal ganglia, enabling direct functional comparisons of homologous circuitry between sexes. Optical stimulation of males or females initiates the unilateral wing vibrations that normally generate the male courtship song. The pattern-generating circuit operates differently in the two sexes, producing wing movement and sound in both but authentic songs only in males and in females expressing male *fru* product. A song-like motor program is thus present in females but lies dormant because the neural commands required for song initiation are absent. Supplying such commands artificially reveals *fru*-specific differences in the internal dynamics of the song generator and sets the stage for exploring their physiological basis.

INTRODUCTION

The males and females of most animal species exhibit profound differences in behavior. Male fruit flies, for instance, court females with a ritual whose central element is a unilateral wing vibration (Sturtevant, 1915; Bastock and Manning, 1955) that produces near-field sound in two modes (reviewed by Tauber and Eberl, 2003). One mode, sine song, is an ~140–170 Hz hum (von Schilcher, 1976b), whereas the other mode, pulse song, consists of brief, repetitive amplitude modulations of an ~150–300 Hz carrier wave (Shorey, 1962; Bennet-Clark and Ewing, 1967; Ewing and Bennet-Clark, 1968). Only male flies sing, and only females respond to song by allowing copulation.

These sex-specific differences in behavior are considered instinctive or innate (Bastock, 1956; Baker et al., 2001); they are thought to reflect genetically determined variation in the structure and/or function of the underlying neural circuits. In *Drosophila*, the expression of many aspects of male or female reproductive behavior results from the action of a single regulatory gene termed *fruitless* (*fru*) (Baker et al., 2001; Demir and Dickson, 2005). The *fru* locus encodes a complex collection of transcription factors (Ito et al., 1996; Ryner et al., 1996) whose expression is restricted to the nervous system (Ryner et al., 1996; Lee et al., 2000; Billeter and Goodwin, 2004; Manoli et al., 2005; Stockinger et al., 2005). Fru isoforms encoded by the P1 transcript exist only in males, due to alternative mRNA splicing (Ito et al., 1996; Ryner et al., 1996). Forcing the expression of male-specifc P1 products (Fru^M) in females causes these females to display male-specific behaviors (Demir and Dickson, 2005).

Fru^M is present in ~2000 neurons (~2% of the entire neuronal population) in the male CNS. Targeted insertion of *GAL4* into the *fru* locus (fru^{GAL4}) has provided genetic access to these neurons and their counterparts in females and revealed their locations in the nervous system (Manoli et al., 2005; Stockinger et al., 2005). Fru^M is found in subsets of sensory, central, and motor neurons, in a distribution suggesting a—perhaps connected—circuit involved in all aspects of male courtship. Surprisingly, however, *fru* neurons exhibit only subtle anatomical differences between the sexes (Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005; Rideout et al., 2007). This creates a considerable mystery: if the male and female nervous systems contain courtship circuitry that is rather similar, what accounts for the dramatic difference in behavior?

Possible solutions to this mystery could take a number of forms. Consider, for example, the pattern generator for song. Although the precise wiring diagram of this circuit in the thoracic ganglion (Hall, 1979; von Schilcher and Hall, 1979) has not been delineated, the song generator likely incorporates Fru^M-expressing neurons at some or all of its key nodes. Intrinsic *fru*-dependent differences in the excitable properties or the synaptic connectivity of these neurons could explain the absence of song in females. Fru could then be viewed as a "transcriptional neuromodulator" whose effect on the circuit is to block out the motor program for song. Alternatively, *fru* control could be extrinsic to the pattern generator itself. Although fully capable of song production, the female circuit would remain silent because descending commands required to call it into action are missing.

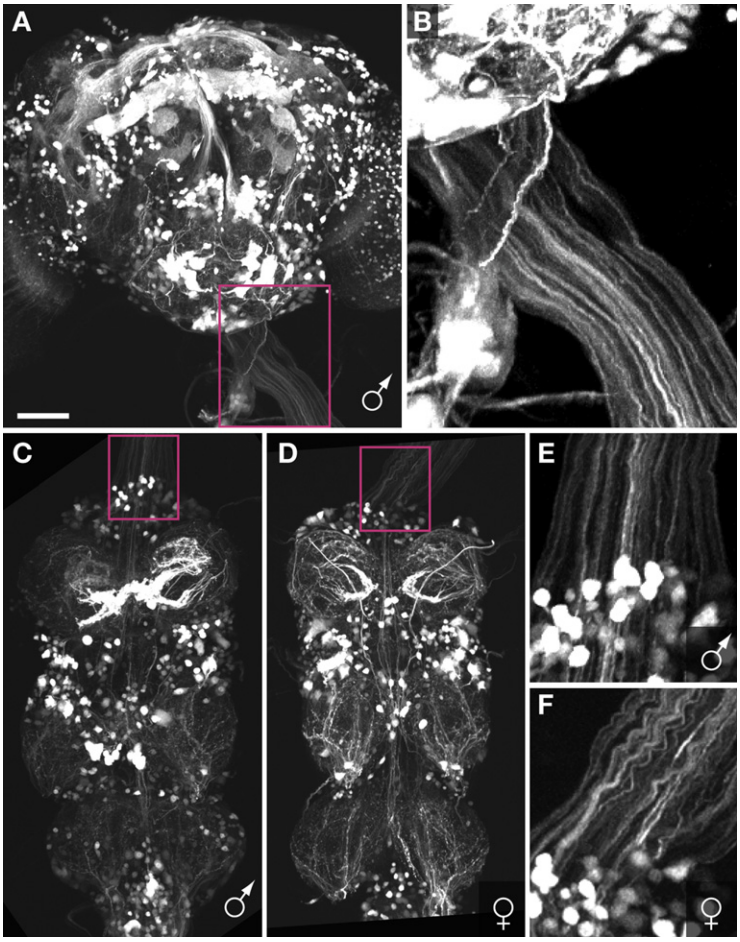

Figure 1. *fru*-Expressing Neurons in the Neck Connectives of Males and Females

(A–F) Neurons expressing Fru^M in males and their counterparts in females are labeled with membrane-bound mCD8-GFP. Brains (A and B) and ventral ganglia (C–F) were dissected 4 days after eclosion, fixed, and labeled with antibodies against GFP. Maximum intensity projections of confocal sections through a male brain (A) and male and female ventral ganglia (C and D, respectively), acquired at an axial spacing of 1 μm, are displayed. Scale bar, 50 μm. Contrast-enhanced images of boxed areas are reproduced at 3× magnification on the right (B, E, and F). Note *fru*-expressing axon fascicles in the neck connectives.

These mechanisms are not mutually exclusive, and classic genetic studies have in fact hinted that they might coexist (Hotta and Benzer, 1976; Hall, 1977, 1979; von Schilcher and Hall, 1979). The superior protocerebrum must be male for unilateral wing extension in gynandromorphs (Hotta and Benzer, 1976; Hall, 1977, 1979); this region might therefore house male-specific "command" neurons (Wiersma and Ikeda, 1964; Hedwig, 2000) responsible for song initiation. Proper song execution has been linked to the presence of male tissue in the thoracic ganglia of gynandromorphs (Hall, 1979; von Schilcher and Hall, 1979). But because these mosaics are composed of many male and female tissue patches throughout the nervous system, these associations are at best indirect.

A direct way to discern intrinsic from extrinsic mechanisms of neural control would be to activate a *fru*-expressing circuit artificially in both sexes and characterize the resulting behaviors. Genetically targeted photostimulation (Zemelman et al., 2002; Lima and Miesenböck, 2005; Miesenböck and Kevrekidis, 2005; Adamantidis et al., 2007; Szobota et al., 2007) of *fru* neurons in the thoracic-abdominal ganglia has allowed us to perform such an experiment. Our analysis focused on the production of the courtship song and yielded answers to two questions. First, is activation of *fru* neurons sufficient for driving courtship behaviors in males? And second, can direct stimulation of *fru* neurons in females produce male-specific behavior, or does the female circuit operate in an intrinsically different mode or not at all?

RESULTS

The putative song generator of male flies is located in the mesothoracic segment of the ventral nerve cord (Hall, 1979; von Schilcher and Hall, 1979). This segment contains ~220–270 and ~200–220 *fru* neurons in males and females, respectively (Billeter and Goodwin, 2004; Stockinger et al., 2005; Rideout et al., 2007). The brain communicates with circuits in the thoracic-abdominal ganglia via several hundred axon tracts bundled in the neck connectives (Coggshall et al., 1973; Borst, 1990). Decorating the cell membranes of *fru* neurons with an mCD8-GFP marker reveals that ~20 cervical fascicles house *fru*-positive axons in both sexes (Figure 1; 20.35 ± 1.42 versus 20.88 ± 1.77 *fru*-positive fascicles in males versus females, means ± SEM, n = 10 flies per sex; p = 0.7334, Wilcoxon rank-sum test). Most of these axons appear to descend, as the number of GFP-labeled tracts remains unchanged when a *teashirt-GAL80* (*tsh-GAL80*) transgene antagonizes fru^{GAL4}*:UAS-mCD8-GFP* expression in the trunk (Fasano et al., 1991; Röder et al., 1992; Calleja et al., 1996; Shiga et al., 1996) (Figure S1; 18.69 ± 1.17 versus 20.62 ± 1.10 *fru*-positive axon fascicles in the presence versus absence of *tsh-GAL80*, means ± SEM, n = 13 and 20 flies, respectively; p = 0.2100, Wilcoxon rank-sum test). Although this figure may be a slight overestimate because *tsh-GAL80* represses fru^{GAL4} activity only partially in some thoracic segments (Figure S1), we draw the tentative conclusion that ~10 pairs of descending interneurons express *fru* in both sexes. These neurons are likely involved in the control of sex-specific behaviors.

The full complement of *fru* neurons was equipped with a light-addressable actuator (Zemelman et al., 2002; Lima and Miesenböck, 2005; Miesenböck and Kevrekidis, 2005) by expressing a *UAS*-driven transgene encoding the ATP-gated cation channel $P2X_2$ (Zemelman et al., 2003) under fru^{GAL4} control (Stockinger et al., 2005). Flies were injected with DMNPE-caged ATP, which could be photolyzed by 100 ms pulses of ultraviolet light. Because *Drosophila* lacks endogenous ATP-gated channels, the photoreleased ATP selectively depolarizes *fru* neurons

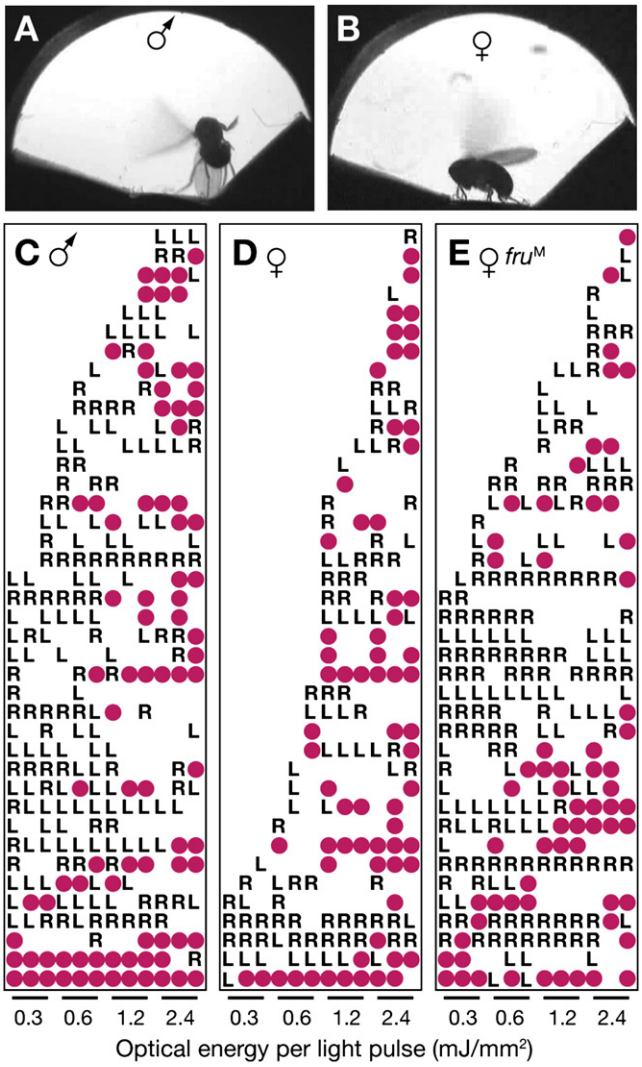

Figure 2. Optical Activation of Unilateral Wing Vibrations

(A and B) Video stills of a headless male (A) and a headless female (B) expressing $P2X_2$ under fru^{GAL4} control. Both flyPods respond to 100 ms pulses of light with unilateral wing vibrations.

(C) Score sheet of photostimulation trials with n = 40 male flyPods. Each row plots the responses of 1 flyPod to 12 successive optical stimuli (three light pulses each at four exponentially escalating energy levels; 15 s between successive pulses). Wing movements were classified as left (L), right (R), bilateral (red circles), or absent (blank spaces). Trials are arranged from bottom to top in descending order of responsiveness.

(D) Score sheet of photostimulation trials with n = 40 female flyPods. Each row plots the responses of 1 flyPod to 12 successive optical stimuli (three light pulses each at four exponentially escalating energy levels; 15 s between successive pulses). Wing movements were classified as left (L), right (R), bilateral (red circles), or absent (blank spaces). Trials are arranged from bottom to top in descending order of responsiveness. Note that the activation threshold for unilateral wing vibrations is shifted toward higher optical energies in females as compared to males.

(E) Score sheet of photostimulation trials with n = 40 female flyPods expressing Fru^M. Each row plots the responses of 1 flyPod to 12 successive optical stimuli (three light pulses each at four exponentially escalating energy levels; 15 s between successive pulses). Wing movements were classified as left (L), right (R), bilateral (red circles), or absent (blank spaces). Trials are arranged from bottom to top in descending order of responsiveness. Forcing the expression of Fru^M in the nervous system of females lowers the optical activation threshold for unilateral wing vibrations from that of wild-type females (D) to that of males (C).

expressing $P2X_2$ (Lima and Miesenböck, 2005). Behavioral responses to the optical stimulus were recorded and scored blind to sex and genotype.

Remote Activation of Male Courtship Songs

By photoactivating all ~2000 *fru* neurons of males at once, we were able to elicit courtship behaviors, such as abdominal thrusting and unilateral wing vibrations, but only in a small fraction of 1.7% of all trials (n = 240). The lack of consistent responses under these circumstances is not entirely surprising: different subsets of *fru* neurons likely play antagonistic roles in courtship (Broughton et al., 2004) so that their simultaneous activation may result in conflicts. However, when the *fru* circuitry of the ventral ganglion was isolated by physically severing the neck connectives, headless male torsos ("flyPods") sang readily and reliably when exposed to light (Figures 2A and 2C, Movie S1).

The females of some insect species decapitate their suitors, possibly to remove descending inhibition and thereby enhance male sexual performance (Roeder, 1935). Playing a similar trick, we managed to kick-start the song generator in 46% of the 219 flyPods tested, with a median optical activation threshold of 0.3 mJ/mm^2 in males (Figure 2C). To discern whether the thoracic circuit was activated directly or indirectly (that is, by synaptic impulses from the distal axonal stumps of descending *fru* neurons), we used a *tsh-GAL80* transgene (Fasano et al., 1991; Röder et al., 1992; Calleja et al., 1996; Shiga et al., 1996) to inhibit fru^{GAL4} and repress expression of the phototrigger in the trunk but not in descending axons (Figure S1). Illumination of *tsh-GAL80*; fru^{GAL4}:*UAS-P2X*$_2$ flyPods at light intensities of up to 2.4 mJ/mm^2 failed to elicit a single wing response in 336 trials (n = 28 headless males). Because *tsh-GAL80* only affects neuronal responses to light but not endogenous electrical activity, this experiment demonstrates that photostimulating the severed axons of *fru*-positive descending interneurons is insufficient to trigger the song generator. The light-activated behaviors we observe therefore reflect the autonomous properties of the thoracic pattern-generating circuit.

Video recordings of optically activated courtship songs document movements that are superficially indistinguishable from those of courting flies: one wing extends from the body at approximately a right angle and vibrates (Figure 2A; Movie S1). In intact flies, some of the asymmetry of wing movement must arise from asymmetric sensory input, as males generally court with the wing that is oriented toward the female (Bastock and Manning, 1955; Ewing and Bennet-Clark, 1968). It is striking that even flyPods sing asymmetrically, relying on one or the other wing during repeated stimulation (Figure 2C).

A flyPod's initial choice of wing appears random, with nearly equal frequencies of left and right extensions (Figure 2C; Table 1). Responses to subsequent stimuli are biased in favor of repeated vibrations of the same wing (Figures 2C–2E); Markov models of wing use yield ~5-fold higher transition probabilities to the ipsilateral versus the contralateral wing (Table 1). Although

Table 1. Patterns of Optically Evoked Wing Movements

	Males	Females	fru^M Females
Probabilities of initial wing choice*			
Left	0.56 (38)	0.49 (18)	0.51 (29)
Right	0.44 (30)	0.51 (19)	0.49 (28)
Transition probabilities during repeated wing use**			
Ipsilateral	0.53 (92)	0.48 (40)	0.61 (105)
Contralateral	0.09 (16)	0.14 (12)	0.06 (9)
Bilateral	0.13 (23)	0.12 (10)	0.14 (24)
Stop	0.25 (44)	0.26 (22)	0.19 (33)

Probabilities of initial and repeated wing use were estimated from the photostimulation trials in Figure 2; numbers in parentheses give the number of events on which these estimates are based. Each continuous run of light-evoked wing movements was treated as an independent stochastic process. Initial responses relied evenly on the left and right wings, but subsequent responses showed a preference for reusing the same (ipsilateral) over switching to the opposite (contralateral) wing. *fru*-dependent differences in wing use patterns are not apparent.

*p = 0.7437 for the null hypothesis of no *fru*-dependent differences in initial wing choice ($\chi^2 = 0.5921$).

**p = 0.1401 for the null hypothesis of no *fru*-dependent differences in transition probabilities during repeated wing use ($\chi^2 = 9.6510$).

physiological mechanisms, such as plateau potentials (Kiehn, 1991; Marder and Calabrese, 1996) or slow inhibition, could explain the observed preference, we cannot exclude that difficult-to-control technical factors, such as the precise orientation of each flyPod in the optical field or the distribution of the injected DMNPE-ATP, also play a role. Despite this caveat, the asymmetry of the movement itself is without doubt a function of the *fru*-expressing circuit and not the method of its activation, as bilateral wing movements are the rule when neurons innervating the flight motor are photostimulated under identical conditions (Lima and Miesenböck, 2005).

The song generator in the thoracic ganglion thus possesses an intrinsic symmetry-breaking mechanism. A likely candidate is reciprocal inhibition between the two hemicircuits on either side of the body. The asymmetry of movement, however, tends to break down under intense optical stimulation, and wing vibrations become increasingly bilateral and flight-like (Figure 2C).

Singing Females

Photostimulation of fru^{GAL4} neurons in headless females was also able to elicit unilateral wing vibrations (Figure 2B; Movie S2), but 4-fold higher photon doses than in males were necessary on average to elicit female "songs" (median activation threshold = 1.2 mJ/mm^2; Figure 2D). The higher energies required to activate the song generator in females could reflect an intrinsic difference in the excitability of the circuit, consistent with a biophysical or wiring effect of *fru* via the gene-expression program it controls. Alternatively, the elevated activation threshold in females could be a trivial consequence of sex differences in either fru^{GAL4} expression levels (and hence, photocurrent amplitudes) or body size: because females are larger than males, fewer photons than in males might penetrate to *fru* targets in the thoracic ganglion, causing these neurons to be activated less efficiently.

To exclude sex-specific variation in fru^{GAL4} promoter strength, nuclear fluorescence intensities were measured in four groups of animals expressing fru^{GAL4}*:UAS–GFPnls* transgenes: in males and females reared at 18°C, and in males and females reared at 25°C. Varying the temperature provides internal validation for these measurements, as transgene expression from the GAL4-UAS system is known to increase at elevated temperatures (Brand et al., 1994). Confocal fluorometry of 480 mesothoracic nuclei in each of the four groups of animals (n = 6–8 flies per group) indeed reported a temperature-dependent rise in fru^{GAL4}-dependent GFP expression but no difference between males and females (p = 0.0003 and 0.2038 for the null hypotheses of no temperature and no sex effects, respectively, Friedman's two-way ANOVA; see Table S1 for detail).

To control for the effect of body size, we forced male *fru* splicing in females (Demir and Dickson, 2005). Because morphological development and sexual behavior are controlled by parallel branches of the sex determination hierarchy (*doublesex* and *fru*, respectively) (Taylor et al., 1994; Baker et al., 2001), a fru^M female harbors a masculinized nervous system in a normal-sized female body (Demir and Dickson, 2005). The photon dose response of headless fru^M females for unilateral wing vibrations matched that of fru^M males, not fru^F females, eliminating body size as a confound (median threshold = 0.3 mJ/mm^2, Figure 2E). Consistent with this interpretation, the median activation thresholds for flight-like responses were identical at 1.2 mJ/mm^2 in males, females, and fru^M females (Figures 2C–2E). Sex-specific differences in excitability are thus particular to the motor program for song.

Acoustic Structure of Male and Female Songs

Audio recordings from male flyPods revealed the characteristic acoustic features of native courtship song, in particular, the presence of sine and pulse episodes (Figures 3A and 3B) (Shorey, 1962; Bennet-Clark and Ewing, 1967; Ewing and Bennet-Clark, 1968; von Schilcher, 1976b; Wheeler et al., 1988; Tauber and Eberl, 2003). Song episodes were detected and classified by measuring the number of zero crossings within sliding 20 ms intervals of voltage-time plots (Figure 3) (Wheeler et al., 1988) and checked against the simultaneous presence of unilateral wing vibrations in the video track.

Sine segments lasted from ~100 ms to several seconds and contained fundamental frequencies of 155 ± 2.40 Hz (mean ± SEM, n = 46; Figures 4A and S2). Pulse trains comprised 4–65 pulses, each containing 2 to 3 damped vibration cycles at 186 ± 4.22 Hz (mean ± SEM, n = 41; Figures 4B, 4C, and S2). The seemingly authentic sonic output of headless bodies demonstrates that much of the "score" for sine and pulse song is encoded in the thoracic circuitry. However, the pulse song of headless males often lacked the metronomic precision and high-pulse repetition rates of courting flies (Shorey, 1962; Bennet-Clark and Ewing, 1967; Ewing and Bennet-Clark, 1968; Wheeler et al., 1988): instead of generating a pulse roughly every 35 ms (corresponding to a repetition frequency of ~28.5 Hz), flyPods emitted at rates between 9.2 and 27.3 Hz (n = 39). The consistently slower natural frequencies of the isolated thoracic circuits suggest that descending inputs from the brain normally synchronize the pulse-song generator to a faster central clock.

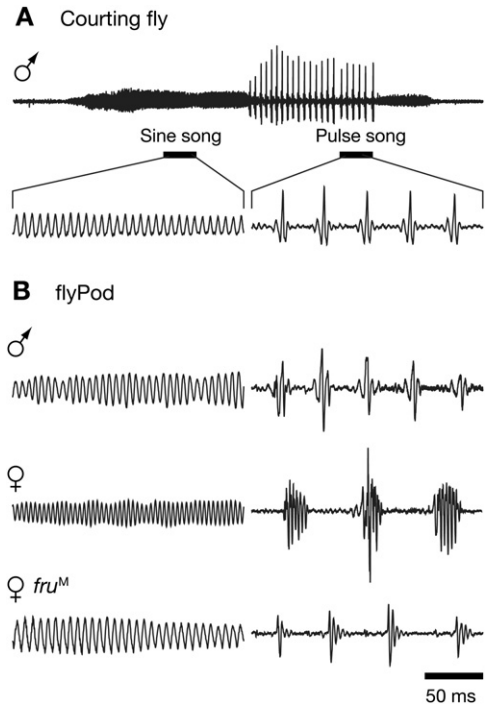

Figure 3. The Light-Activated Song Contains Sine and Pulse Elements

(A) Voltage-time plot of the native song of a Canton S male courting a virgin female. Sine and pulse song segments, each ~200 ms in duration, are reproduced at an expanded timescale below. In this example, the sine song frequency is 157 Hz, and the interpulse interval averages 38 ms.

(B) Voltage-time plots of light-activated flyPod songs. flyPods of either sex generate sine and pulse songs upon optical stimulation. Male flyPods reproduce the sine-song frequency and pulse waveform of native courtship song (A), whereas female flyPods do not. Forcing the expression of *fru*^{M} sets the female circuit to male mode.

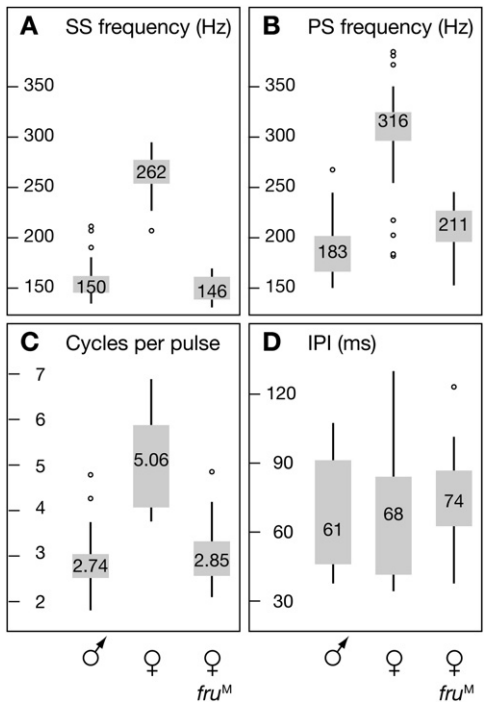

Figure 4. *fru* Determines the Acoustic Structure of the Light-Activated Song

(A–D) Box plots of (A) sine-song (SS) frequency, (B) pulse-song (PS) intrapulse frequency, (C) PS cycle number per pulse, and (D) interpulse interval (IPI) indicate population medians numerically, interquartile ranges by gray boxes, ranges by whiskers, and outliers by open circles. Sine-song frequency, pulse-song frequency, and pulse-cycle number of females differ significantly from those of males and *fru*^{M} females ($p < 0.0001$, Kruskal-Wallis ANOVA; see Table S2 for statistical detail).

We conclude that signals from the brain are important for initiating sine and pulse song but largely dispensable for its execution. This division of labor is not surprising, given that ten pairs of descending interneurons form the putative link between courtship circuitry in the fly's head and its body (Figure 1). Behaviors unfold as these descending interneurons call on largely autonomous motor circuits in the ventral nerve cord (Wiersma and Ikeda, 1964; Marder and Calabrese, 1996; Hedwig, 2000).

Sound produced by headless females vibrating one wing was less cleanly structured than that of males, often lacking a stereotyped pulse waveform (Figure 3B), sufficient dampening to limit pulses to three vibration cycles (Figures 4C and S2), and stable sine frequencies below the ~220 Hz flight oscillation (Figures 3B, 4A, and S2; see Table S2 for statistical analysis). Spontaneous transitions to bilateral wing movements and high-intensity buzzing were common, consistent with the small separation of the optical activation thresholds for the two motor programs in females (Figure 2D).

To determine whether differences in acoustic output were due to differences in the neural control or the mechanics of sound production, we studied *fru*^{M} females (Demir and Dickson, 2005), in which masculinized neurons act on a female musculoskeletal system and wing. These *fru*^{M} females produced male songs (Figures 3B, 4A–4D, and S2; see Table S2 for statistical analysis): a human observer analyzing audio recordings in a blind manner classified songs according to the *fru* allele that was expressed ($\chi^2 = 13.7647$; $p = 0.0002$), not according to the sex of the organism that expressed the allele ($\chi^2 = 0.1955$; $p = 0.6584$).

Reciprocal experiments with *fru*^{F} males (Demir and Dickson, 2005) yielded ambiguous results. *fru*^{F} males generated sine and pulse songs, but the acoustic characteristics of these songs showed broad bimodal distributions, with peaks near the typical male and female modes (Figure S2). These animals thus appear to represent a mixed population, perhaps due to the incomplete penetrance of the *fru*^{GAL4}/*fru*^{F} genotype in males.

Females Sing out of Tune

To assay whether flies were able to distinguish the light-activated songs of male and female flyPods, female virgins were paired with wingless—and therefore mute—males. Because the courtship song is important for female receptivity and male arousal, such couples rarely proceed to copulation (Sturtevant, 1915; von Schilcher, 1976a; von Schilcher, 1976b). The courtship arrest, however, can be overcome by playing back the recorded song of a courting male (Figure 5) (Bennet-Clark and

Ewing, 1967; von Schilcher, 1976a). A wingless male's copulation attempts during song playback thus provide an index of the male's perception of the song and its effects on the female, whereas the willingness of a virgin female to mate with a mute suitor reflects a song's authenticity largely through female "ears."

Our playback experiments compared the efficacy of native courtship songs in promoting mating behavior with that of the light-activated sonic output of males, females, or fru^M females. Experimental soundtracks consisted of repeating 2 s audio samples. Each sample contained either 500 ms of sine song, 500 ms of pulse song, or a concatenation of both song types in experimental background noise. Fly pairs—a wingless male and a virgin female—were allowed to interact in silence for 5 min (white backgrounds in Figure 5B) and were then exposed to sound for 10 min (gray backgrounds in Figure 5B). Samples of the light-activated sine and pulse songs of headless males and fru^M females (Movie S3), but not those of wild-type females (Movie S4), substituted fully for the songs of courting males (Figures 5A, 5B, and 5C). The pulse songs of male or female flyPods expressing fru^M could by themselves drive courtship as efficiently as native songs (Figures 5B and 5C). In contrast, all light-evoked sound emissions from wild-type females proved ineffective (Figures 5A, 5B, and 5C; $p < 0.005$, Kruskal-Wallis ANOVA; see Table S3 for statistical detail). A song generator configured by *fru* to run in male mode thus produces essentially authentic courtship song, whereas a female-configured circuit does not.

DISCUSSION

The capacity to activate the pattern generator for song at will, outside the behavioral context of courtship, in females as well as in males, has uncovered four functional circuit states that give rise to four types of sonic output: the sine and pulse songs of males, and their recognizable—but also recognizably distinct—equivalents in females (Figures 3–5). Whereas male songs are integral parts of *Drosophila's* natural courtship, the female songs recorded here are heard only outside normal physiological limits: they require an artificial trigger, which supplies an initiating command that the female nervous system does not normally provide.

Neural Control of Song Production in Males and Females

The absence of courtship song in females is thus not due to the absence or inactivity of the actual motor program for song. Rather, it reflects control at a hierarchically higher level, extrinsic to the song generator itself. Differences between the sexes could, for example, arise because central circuits involved in action selection process sensory cues differently in males and females, or because these circuits relay their output onto different sets of descending interneurons. A key site for the sex-specific control of song production may indeed be these "command" neurons themselves. *Drosophila* possesses ~10 pairs of descending axon tracts containing *fru*-positive processes (Figure 1). Male-female differences in the number of these tracts are not apparent, counter to the idea of a simple dimorphism in which the neurons required to relay central commands for male-specific behaviors to thoracic motor circuits exist only in males.

Males do, however, harbor two dozen mesothoracic neurons whose specification depends on the male-specific forms of both *fruitless* and *doublesex*; these cells are therefore absent in wild-type and fru^M females (Rideout et al., 2007). It appears likely that these sexually dimorphic neurons form a critical conduit between descending interneurons and the song generator. We know that fru^M females are fully capable of producing authentic male songs when their thoracic *fru* neurons are directly activated by light (Figures 3–5). Yet spontaneous courtship songs in these animals are infrequent and, in the rare instances when they do occur, telegraphic (Rideout et al., 2007). Taken together, these pieces of evidence suggest that male-specific neurons couple the song generator to descending commands controlling song initiation and pulse emission, and that this link is broken or poorly transmissive in wild-type and fru^M females.

The discovery of a latent motor program for song in female flies resonates with a recent observation in mice: silencing or ablating pheromonal inputs causes the derepression of male sexual behaviors in females (Kimchi et al., 2007). Blocking pheromonal inputs in female flies, in contrast, fails to unmask male-specific behaviors (Stockinger et al., 2005; Kurtovic et al., 2007). This is consistent with the idea that female flies, rather than actively repressing male behaviors like mice, lack the commands to activate them. Despite the inverted logic, a common principle is thus beginning to emerge from studies of two evolutionarily distant species: sex-specific behaviors may be controlled by a handful of master neurons that recruit or repress, in a sex-specific manner, subordinate effector routines that are present and functional in both sexes. In other words, setting a few critical switches in an otherwise largely "unisex" system to male or female mode may suffice to generate the fundamental behavioral differences between the sexes. This would represent an elegant alternative to building entirely separate neural substrates for male and female behaviors into each developing nervous system.

An important qualification, however, applies. Although the song generator is functional in both sexes, we detect clear differences in the way the circuit operates in males and females (Figures 2–5). The activation threshold of the female circuit is higher than that of the male song generator (Figure 2); the frequencies of female sine and pulse songs exceed the respective frequencies in males (Figures 3 and 4); and the female pulse generator is poorly dampened, adding a variable amount of ringing to the precise three oscillation cycles of males (Figures 3 and 4). Because these intrinsic functional characteristics of the pattern generator are directly determined by the presence or absence of Fru^M (Figures 3 and 4), they provide a first glimpse of the physiological consequences of sex-differential gene expression in *fru* neurons. Targeted recordings from these neurons during light-activated song will uncover the biophysical properties that provide the circuit and its constituent neurons with their distinct, sex-specific modes of operation. Once the genes regulated by Fru^M have been discovered, these insights promise to merge into a complete molecules-to-systems description of the sex-specific control of a complex innate behavior.

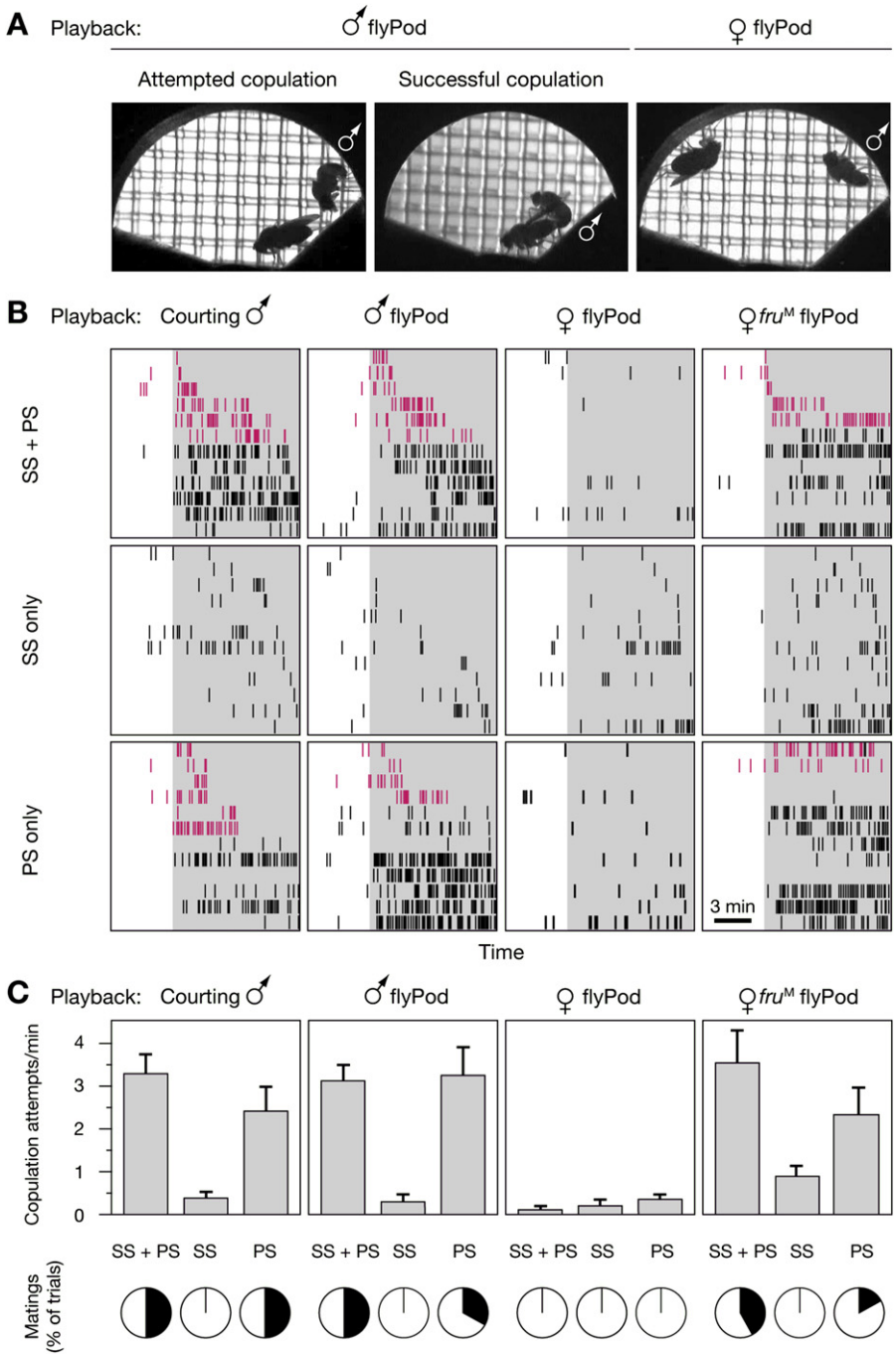

Figure 5. flyPods Expressing *fru*^{M}, but Not *fru*^{F}, Produce Effective Mating Signals

(A) A mute, wingless male is paired with a virgin female in a mating chamber covered with nylon mesh and placed over a loudspeaker. Playback of the light-activated song of a male flyPod stimulates copulation attempts (i.e., abdominal curling) by the wingless male (left panel). These attempts eventually lead to successful mating with the female (i.e., male and female remain in copulation position for >60 s; center panel). Playback of the light-activated song of a female flyPod has no effect on mating behavior (right panel).

(B) Rasters of copulation attempts by individual wingless males during playback of audio signals recorded from four types of sources: Canton S males courting virgin females (left column), male flyPods (second column from left), female flyPods (second column from right), and female flyPods expressing Fru^{M} (right column). Audio samples contained sine- and pulse-song segments (SS + PS; top row of plots), sine song alone (SS only; center row of plots), or pulse song alone (PS only; bottom row of plots). The rasters show, for each type of song, the effects of 12 audio samples on the timing and frequency of male copulation attempts in 12 individual pairings. Behavior was analyzed, blind to the experimental condition, during 5 min of silence (white backgrounds) and 10 min of song playback (gray backgrounds). Trials in which the wingless male mates with the female are indicated in red.

Neural Control of Sine and Pulse Song

Sine and pulse song are examples of two fundamental forms of periodic behavior: quasilinear and relaxation oscillations (van der Pol, 1926; Hirsch and Smale, 1974; Strogatz, 1994). Quasilinear oscillators generate sine waves, whereas relaxation (or integrate-and-fire) oscillators "relax" strain accumulated during phases of slow buildup (here, the interpulse intervals) in abrupt discharges (here, the pulses of pulse song). Because remote activation of all thoracic *fru* neurons can elicit either sine or pulse song (Figure 3), the generators for both types of song must contain (or be connected to) *fru*-positive cells. But what is the precise relationship between these song generators? Are sine and pulse song the products of separate, mutually inhibitory circuits? Or, can the same pattern generator toggle between two distinct functional modes?

Circuits capable of switching between quasilinear and relaxation oscillations indeed exist. The classical example is the electronic triode oscillator studied by van der Pol in the 1920s (Appleton and Van der Pol, 1922; van der Pol, 1926, 1934). Depending on the value of a single parameter, which characterizes the nonlinearity of the triode's resistance, the van der Pol circuit oscillates in either sine or "pulse" mode (van der Pol, 1934). It is conceivable that the song generator of the fly contains a biophysical equivalent of the triode's resistance. One type of descending interneuron could then set the value of this parameter and thereby control the type of acoustic output (sine or pulse song), whereas a second class of interneuron could signal the decision to sing, without specifying a particular song rhythm.

An organization of the pattern generator into separate circuits for sine and pulse song would require a different command structure. Again, a minimum of two classes of descending interneurons are necessary. But instead of one neuron serving as an on-off switch and the other as a selector of song type, the two classes of neurons now function independently; they form "labeled lines" for controlling sine and pulse song, respectively. Our current data do not allow us to distinguish between these organizational models. That said, the seamless transitions between sine and pulse rhythms that characterize the native song (see, for example, Figure 2A) would appear to favor the existence of a single, continuously running pattern generator that switches smoothly between quasilinear and relaxation modes.

Definitive insight into the problems of rhythm control and sex-specific song production will require that the relevant command neurons are identified and activated individually. Progress toward this goal will also bring us one essential step closer to unraveling the central circuits that regulate the activity of the descending interneurons themselves.

EXPERIMENTAL PROCEDURES

Fly Strains

Flies were cultured at 25°C on standard food on a 12 hr:12 hr light:dark cycle. The sexes were separated within 12 or 4 hr of eclosion for photostimulation and song-playback experiments, respectively, and aged for ≥4 days before testing. Flies used in photostimulation experiments were hemizygous at the *fru* locus, carrying fru^{GAL4}, *UAS-P2X*$_2$ on the paternal III chromosome (Lima and Miesenböck, 2005; Stockinger et al., 2005) and fru^{M}, fru^{F}, or the wild-type *fru* allele on the maternal III chromosome (Demir and Dickson, 2005), or vice versa. Strains for anatomical studies harbored fru^{GAL4} and *UAS-GFPnls* or *UAS-mCD8-GFP* transgenes. Where indicated, experimental strains also carried a *tsh-GAL80* transgene (the kind gift of Julie Simpson), which was generated by targeted transposition of the *tsh-GAL4* line (Calleja et al., 1996; Shiga et al., 1996).

flyPod Song

Flies were briefly iced and decapitated. The exposed nerve cord was injected (Nanoject II, Drummond) with 18.4 nl of 60 mM DMNPE-ATP (Molecular Probes) in artificial hemolymph (5 mM Na-HEPES, pH 7.3, 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM trehalose, 10 mM sucrose) (Lima and Miesenböck, 2005). Injected flyPods were allowed to recover for >5 min in a humidified chamber and tested within 30 min. Single flyPods were positioned near an NR3160 miniature velocity microphone (Knowles Acoustics) in a 10 mm cylindrical arena with quartz glass floor and ceiling. The arena was mounted within a light-proof, anechoic enclosure and viewed under 950 nm LED light (TSAL6100, Vishay) by a Sunell SN-425M CCD camera equipped with a C-mount macro zoom lens (Navitar Zoom 7000). For optical stimulation (Lima and Miesenböck, 2005), a Q-switched, frequency-tripled Nd:YVO_4 laser emitting at 355 nm (DPSS Lasers, model 3520-30) was switched and intensity-modulated with an acousto-optic deflector (IntraAction model ASN-802832 with ME-802 driver). The modulated beam was expanded, routed into the enclosure, and merged with the infrared illumination path via a UV cold mirror (UR-2x2, CVI).

Video images were captured by a National Instruments PCI-1407 image acquisition board and recorded in MPEG4-compressed format by a virtual instrument written in LabVIEW 7.1 (National Instruments). Audio signals were amplified with a 10,000× instrumentation amplifier (Analog Devices AD620), filtered at 10 kHz (8-pole Bessel, Frequency Devices 9002), digitized with 16 bit precision at 44.1 kHz (M-Audio Delta 66), and recorded in Audacity 1.2.5, an open-source sound editor (http://audacity.sourceforge.net). An audible light cue, presented at the beginning of each experiment, synchronized the video and audio tracks.

Videos were analyzed in VirtualDub 1.6.17 (http://www.virtualdub.org) and scored blind for uni- and bilateral wing extensions initiated within 4 s of each optical stimulus. Sine and pulse song was detected in a semiautomated fashion by measuring the number of zero crossings within sliding 20 ms intervals of voltage-time plots (Wheeler et al., 1988) and checked against the simultaneous presence of unilateral wing vibrations in the video track. Sine and pulse song frequencies were estimated from power spectra of extended sine song episodes (~100–2000 ms) or individual pulses (~12–22 ms), using routines in MATLAB 7.4 or Audacity 1.2.5.

Song Playback

Male virgins were iced and dewinged >1 day before pairing with female virgins. Pairs (age 7 days) were placed in the arena used for photostimulation experiments, but with the quartz glass floor replaced with fine nylon mesh, and allowed to acclimate for 5 min. If males attempted to copulate more than three times during this period, the pair were excluded from the analysis (7% of all flies tested). After acclimation, pairs were exposed for 10 min to a continuously repeated 2 s audio signal played through computer speakers (Companion II, Bose) positioned under the chamber. The signal was monitored by an NR3160 miniature velocity microphone (Knowles Acoustics) in the chamber and adjusted to match the volume generated by courting flies. Each 2 s audio signal contained 500 ms of sine song, 500 ms of pulse song, or 500 ms of sine

(C) Summary statistics of playback experiments. Column plots show the average frequencies of male copulation attempts during song playback until mating (means ± SEM, n = 12 pairings); for trials where mating was delayed beyond 10 min, the entire playback period was scored. Pie charts depict the percentages of trials leading to copulation within 10 min. The frequency of copulation attempts during playback of experimental background noise was 0.28 ± 0.11 per min (mean ± SEM, n = 12 pairings), the percentage of matings 0.

song followed by 500 ms of pulse song in experimental background noise, or noise alone ("silence"), in the frequency band from 50 Hz to 3 kHz.

Confocal Microscopy

Brains and ventral ganglia of flies aged 4 days and expressing *UAS-mCD8-GFP* or *UAS-GFPnls* under *fru*^{GAL4} control were dissected in artificial hemolymph and fixed for 20 min in 4% (v/v) paraformaldehyde plus 0.01% (v/v) Triton X-100 in 100 mM phosphate buffer (PB), followed by three 10 min washes in PB. For immunolabeling where indicated, specimens were permeabilized with 0.2% Triton X-100 in PB (PBT) during washing and blocked with 5% goat serum in PBT for 1–2 days at 4°C. Specimens were labeled overnight with rabbit polyclonal antibodies against GFP (1:100). Bound antibodies were detected with secondary AlexaFluor-488 conjugates (Molecular Probes) at 1:500 dilution. Specimens were mounted in VectaShield with DAPI (Vector Laboratories). Image stacks with an axial spacing of 1 μm were collected on a Zeiss LSM 510 confocal laser-scanning microscope, analyzed in Image J 1.37 (http://rsb.info.nih.gov/ij), and rendered in Imaris 4.0.1 (Bitplane).

SUPPLEMENTAL DATA

Supplemental Data include two figures, three tables, and four movies and can be found with this article online at http://www.cell.com/cgi/content/full/133/2/354/DC1/.

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